Microbiology and Molecular Biology Tools for Biogas Process Analysis, Diagnosis and Control

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Abstract Many biotechnological processes such as biogas production or defined biotransformations are carried out by microorganisms or tightly cooperating microbial communities. Process breakdown is the maximum credible accident for the operator. Any time savings that can be provided by suitable early-warning systems and allow for specific countermeasures are of great value. Process disturbance, frequently due to nutritional shortcomings, malfunction or operational deficits, is evidenced conventionally by process chemistry parameters. However, knowledge on systems microbiology and its function has essentially increased in the last two decades, and molecular biology tools, most of which are directed against nucleic acids, have been developed to analyze and diagnose the process. Some of these systems have been shown to indicate changes of the process status considerably earlier than the conventionally applied process chemistry parameters. This is reasonable because the triggering catalyst is determined, activity changes of the microbes that perform the reaction. These molecular biology tools have thus the potential to add to and improve the established process diagnosis system. This chapter is dealing with the actual state of the art of biogas process analysis in practice, and introduces molecular biology tools that have been shown to be of particular value in complementing the current systems of process monitoring and diagnosis, with emphasis on nucleic acid targeted molecular biology systems.

Keywords Biogas process parameters · Molecular biology tools · Quantitative Real-Time PCR · Next generation sequencing · Meta-omics · Fluorescence-in situ hybridization · Metabolic quotient

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Abbreviations

BB	Bead-beating
BMP	Biological/biochemical methane potential
BLAST	Basic local alignment search tool
Bp	Base pair(s)
cDNA	Complementary DNA (transcribed from RNA species)
CLSM	Confocal laser scanning microscopy
COD	Chemical oxygen demand
DGGE	Denaturing-gradient gel electrophoresis
DNA	Deoxyribonucleic acid
FISH	Fluorescence in situ hybridization
LCB	Lignocellulosic biomass
LM	Light microscopy
MQ	Metabolic quotient
mRNA	Messenger RNA
NA	Nucleic acid(s)
NGS	Next generation sequencing
OLR	Organic loading rate
PC(o)A	Principal coordinate/Principal component analysis
PCR	Polymerase chain reaction
PSM	Process simulation model
qPCR	Quantitative Real-Time PCR
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcription
SCFA	Short-chain fatty acid(s) or also VFA
SEM	Scanning electron microscopy
SMA	Specific methanogenic activity
TEM	Transmission electron microscopy
TGGE	Temperature-gradient gel electrophoresis
TVA/TIC	Total volatile acids/total inorganic carbon
VFA	Volatile fatty acids
VOA	Volatile organic acids

Contents

1 Introduction

Biogas production by anaerobic digestion of organic matter is a bio-technology with very long tradition for some 2,000–3,000 years. It was applied initially for sanitation purposes and only later additionally for energy production. The issue sanitation with its beneficial effects for the society is presented within this book in Chap. 3. All of the process steps are performed in a food chain by different microorganisms, governed by process engineering in a suitable technical environment. Some of these microbes have to cooperate extremely efficiently in syntrophic dependency in order to be able to thrive and proliferate at the minimum limit of possible energy gain [1, 2].

Methanogenic archaea, and among these particularly the acetoclastic *Methanosaetaceae*, appear to be most sensitive in biogas processes to stress factors such as short retention times, high ammonia, oxygen and short-chain fatty acid (SCFA) concentration, lack of certain trace elements and increased temperature [3, 4]. Due to their relatively low apparent maximum turnover number (K_m) for acetate and long doubling times [5], the acetoclastic methanogens are disfavored at short retention times and increasing acetate concentration in the fermenter [6]. They are increasingly washed out if their proliferation cannot compensate out-dilution. This effect is even pronounced at additional stress conditions, favoring the activity and growth of syntrophic associations with hydrogenotrophic methanogens to the detriment of active *Methanosaetaceae* and acetoclastic activity [3, 4, 7]. It is incorporated as a central point in the bioindicator concept of process diagnosis [4] (see also Sects. 3 and "Microbial Guilds, Bioindicators and Transcriptional Profiling").

The second bottleneck is the thermodynamically difficult hydrogen, formate or electron-releasing conversion of short chain fatty acids (SCFAs), alcohols and other intermediates of the biogas process. Most of these reactions are endergonic at standard conditions but can be realized by syntrophic associations involving product-scavenging methanogens [8, 9]. Methanogenic archaea are able to remove the reaction products by converting them finally to biogas, predominantly CH_4 and CO_2, which segregates from the fermenter sludge to the gas headspace and is further withdrawn by gas utilization. Syntrophic bacteria or anaerobic fungi partners of methanogens are difficult to cultivate and to study without their product-consuming associate. Modern characterization is typically initiated by genome or metagenome analysis, possibly leading to insights about special requirements that allow cultivation of pure isolates and studying their special physiological performances [10–12].

A third recognized bottleneck is the initial rate-limiting hydrolysis of recalcitrant substrates such as lignocellulose-rich biomass (LCB). When compared to aerobic degradation of lignocellulose, considerably less is known on the corresponding anaerobic process and the organisms involved. Besides bacteria, other organisms such as anaerobic fungi may be involved in efficient initial LCB attack and degradation [13]. Chapter 2 in this book is dedicated to anaerobic fungi and recent perceptions of their role in anaerobic LCB digestion. For some of these cellulolytic organisms, the genome has been sequenced [14, 15]. Such genome information is an invaluable data basis for process optimization and further biotechnological exploitation.

Microbial processes in anaerobic digestion are driven by both, biotic and abiotic factors. The physical and chemical environment (e.g. nutritional factors and redox status) are basic to and determine the biotic activity, the substrate conversion by the microbes. Biotic measures to regulate the process (e.g. bioaugmentation) however are scarce as briefly discussed in Sect. 2.7.

The most important issue in process optimization is to avoid the worst case, process disturbance or even breakdown. This requires a process control strategy that includes reliable process diagnosis based on meaningful analytical data. Since the activity of bioindicator microbes, organisms that are typical for certain process conditions, does react before conventionally used process chemical parameters indicate process failure, a promising approach for successful process control is to assess the activity of these bioindicators as integral part of an early-warning system [4]. The relevant actors, i.e. bioindicators performing the crucial biogas process steps, must hence be identified, and suitable analysis tools must be used or developed to track these key organisms and their activity quantitatively.

In the following chapters, microbiology and molecular biology tools for biogas process diagnosis and control are compiled and discussed. Since several important process dynamics such as SCFA and total solid (TS) turnover as well as gas quality/quantity are the result of microbial activity, and respective wet chemistry and physico-chemical analyses are and will be indispensable part of conventional practice but have revealed limitations, recent experience with these conventional applications for agricultural single-stage biogas processes is presented in the following Sect. (2). Molecular biology approaches have only recently emerged and may be introduced into practice after comparison or along with established physico-chemical routines. Some of these molecular tools, however, are promising candidates to be implemented in a holistic suite of analytical tools for process diagnosis and control.

2 Physico-Chemical and Biochemical Process Parameters

The spectrum of physico-chemical parameters actually employed for process diagnosis of agricultural and category 2 biowaste (untreated non-infectious to humans, animals or plants), biogas plants has originally been adopted from anaerobic sewage sludge digestion. Many of these parameters and respective

benchmarks are listed and discussed in a review by Weiland [16]. Important aspects for diagnosis and control of single-stage processes are presented in the following.

2.1 Gas Production

In order to evaluate the efficiency of the process it is indispensable to determine the volumetric gas production and the gas quality or at least estimate these parameters from the generated electricity and the actual adjustment of the combined heat and power unit. Different equipment is on the market, ranging from simple manually operated lab instruments to fully automated industry scale online devices. Combined with data on the fed organic dry matter (oDM or volatile solids [VS]), the gas production and quality data inform on the specific methane production or methane yield (m^3 CH$_4$/kg VS). Comparison with benchmarks for given substrates and interpretation of the recent methane yield development allows to estimate the actual process efficiency at least roughly and to reveal up- and downward trends. Particular attention should be paid to decreasing CH$_4$ and increasing H$_2$ concentrations in the produced biogas. CH$_4$ concentrations falling below ca. 48 % and H$_2$ concentrations exceeding ca. 100 ppm in single stage processes are alarming and should give rise to counteractive measures.

Isotope ratio mass spectrometry (IRMS) based methods analyzing isotope discrimination by the biogas producing microbial community were recently proposed to detect methanogenic pathway shifts [17, 18]. The switch from acetoclastic to hydrogenotrophic methanogenesis is interpreted as signal of stress conditions which may allow plant operators to adjust their feeding strategy. Since the IRMS equipment is expensive, a laser-assisted online analysis technique was described for this purpose as a more practice-oriented alternative [19]. However, a pathway shift does not necessarily indicate imminent process failure, and interpretation problems with data from variable feedstock composed of C4 and C3 plant material still need to be resolved. Although gas analysis using stable isotope ratios has potential to reveal biogas production pathway changes, and online monitoring is possible, its contribution to process diagnosis is thus confined to basic research in its current state.

2.2 Process Intermediates, SCFA, Total and Volatile Solids, and Specific Determinants

The determination of SCFA (also referred to as volatile fatty acids [VFA], or volatile organic acids [VOA]) is a highly important component of process diagnosis. The SCFA spectrum is typically assessed using liquid or gas chromatography (LC/GC) based routines on suitable extracts in an external specialized lab and should include the iso-forms of butyrate and valerate. Increased levels of these

SCFA exceeding ca. 50 mg/L as well as propionic acid concentrations above ca. 1 g/L along with a propionic/acetic acid ratio >1 typically indicate process disturbance in single-stage systems [16], but exceptions have become known as well. Reduced activity of methanogens due to substrate overload or limited availability of essential nutrients such as trace elements is frequently the reason, giving rise to "acid jam", i.e. accumulation of upstream produced intermediates.

Attention must also be paid to the development of the dry matter (or total solids [TS]) content in the digester. TS can easily be determined on-site in an oven at 105 °C, whereas for analysis of VS, a muffle furnace is required. Information on VS contents in the substrates is important for determining the methane yield, and the VS/TS trend in the digester can anticipate eventually problematic ash accumulation. Increasing TS values over time in the fermenter sludge indicate a problem at the hydrolysis/acidogenesis step leading to compromised process efficiency and incomplete digestion. TS values exceeding 15 % can lead to stirring problems in conventional continuously stirred tank reactors (CSTRs). Reducing the organic loading rate (OLR), i.e. increasing the hydraulic retention time (HRT), might be helpful, otherwise substrate conditioning by physical/mechanical or (bio)-chemical means could be considered. More specific information of TS and VS in the fermenter sludge can be obtained by the fractionated analyses according to Weende and Van Soest [20].

Recently, an online near-infrared spectroscopy (NIRS) application to evaluate the process state with potential for the practice was reported [21]. The authors presented acceptable estimations of VS, ammonium, total inorganic carbon (TIC) and total VFA even in short-term process dynamics of a mesophilic pilot-scale maize silage fed biogas digester. Further extension of the model to include different substrates and analysis parameters, and experience in long-term operation is needed before online NIRS systems can be recommended for process control.

Similarly, techniques involving flow-assisted cell sorting (FACS) [22], matrix-assisted laser desorption/ionization—time of flight mass spectrometry (MALDI-TOF/MS) and Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR/MS) [23, 24] as well as secondary-ion mass spectrometry (SIMS) based systems [25, 26] can be helpful to separate or identify distinct microbes or consortia, or track their specific metabolic activities. Such methods prove to be useful in basic microbiology research and taxonomy and allow identifying microbes with resolution at the subspecies or strain level [23], given a suitable reference database is available. However, "dirty" environmental samples can pose considerable problems. Although there is some potential, application for monitoring the process status of the black-box biogas fermenter actually does not appear to be a realistic option. Physical methodologies basing on or coupled with fluorescence-in situ-hybridization (FISH) are itemized in Sect. 3.2.1.

2.3 Early Warning—The TVA/TIC Ratio

The ratio of total volatile acids to total inorganic carbon (TVA/TIC, also referred to as FOS/TAC or VOA/TIC) is determined by 2-point titration (pH 5.0, pH 4.4) and can easily be performed on-site [27]. It beats out the pH value as early indicator of process acidification due to its much higher sensitivity. Currently, the TVA/TIC ratio is the most used process chemical early warning system of acidification. TVA/TIC ratios of 0.15–0.45 are typical for a stable process without major acid accumulation, whereas rising ratios exceeding 0.45 reflect process disturbance, and values above 0.6–0.7 indicate acidosis. This can be associated with TIC depletion, e.g. in case of trace element deficiency.

However, in cases of atypical process conditions such as at higher NH_4^+ concentrations and pH-values, considering only TVA/TIC as a process indicator can be risky. The NH_4^+/NH_3 buffer system can trap protons masking possible acidification events. Obtaining low TVA/TIC values can thus be misleading at higher free ammonia-nitrogen (FAN; see also Sect. 2.4) if distinct SCFA (possibly not measured) may already be at alarming level. Above ca. 1 g NH_4^+-N/L, it is therefore suggested to monitor the SCFA spectrum and/or molecular biology parameters (see Sect. 3) as well in order to perceive process perturbation and acid build-up.

2.4 Nutrients, Toxic and Disturbing Agents

Since nutrient composition of the substrates governs and limits microbial process performance, respective analysis should be performed occasionally and particularly if a plant is operated with atypical substrates or such operation is planned. Modern elementary analysis involves Inductively Coupled Plasma (ICP) equipment with detection by Optical Emission Spectroscopy (OES) or even more sensitive Mass Spectrometry (MS). Experience from practice suggests that the C:N ratio should be about 15–45 and the C:N:P:S ratio about 300-600:15:5:3 [4, 16, 28].

Several toxic agents are known that can impede the anaerobic digestion process. One of the most relevant is free ammonia [29] (see also Sect. 2.3) which can be calculated from the NH_4^+-N concentration, the process temperature and the pH value [30]. Nitrogen seems to be lacking only in exceptional cases, but reduced N-compounds typically accumulate in anaerobic digestion of protein-rich feedstock and can become toxic [6]. It is thus important to determine these parameters periodically. NH_3 diffuses unspecifically inside the cell, can capture protons and hamper proton-dependent ATP generation leading to activity loss and possibly cell death. The typical ammonia toxicity threshold is about 400–500 mg NH_3-N/L, but a higher margin is possible in case of adaptation [4, 29]. Particularly microbes relying on H^+ pumps are susceptible whereas those with Na^+-pumps are favored in the presence of sufficient Na^+. Predominantly microbes involved in the hydrogenotrophic metabolism of biogas intermediates are using Na^+ pumps for ATP

generation [5, 9]. Na^+ appears to limit methanogenesis and elicit acidification at values decreasing to about 10 mg/L in the fermenter sludge and must therefore be provided at higher concentration in the substrate mix [31]. This can explain stabilizing, stimulating effects of Na^+ addition at limiting, constrained or stress conditions such as in high-performance biogas production from grass silage in the practice [4].

Requirement for trace elements (TEs) in biogas production from biomass, particularly of Co, Ni, Se, Fe, and possibly of Mo, B and W, has been described in many publications, e.g. [16, 32–34] and in Chap. 7 of this book. Since their presence in suitable, available concentrations is a precondition of efficient process performance, TE contents should be determined occasionally and particularly if the feedstock composition is changed. Trace elements, however, can become toxic in higher concentration. Other compounds with toxic or disturbing potential are found among e.g. antibiotics, mycotoxins, detergents and heavy metals [35, 36], and some phenolic compounds appear to have inhibitory properties [37]. Cu and Zn loads are of particular importance for agricultural biogas plants. They can originate in higher concentrations from animal husbandry, and according to several practice reports, can be the cause of process disturbance and efficiency loss [38].

Several devices allow the measurement of O_2 and H_2S in the biogas. O_2 can enter the process by leakages or actively during biological desulfurization. CO_2 reduction becomes unfavorable in the presence of better electron scavengers such as O_2, SO_4^{2-} and NO_3^- [6]. The redox potential is increased in their presence, and the activity of most anaerobic microbes is impeded. O_2 should therefore be kept below 1 % (better 0.1 %) in the gas phase, and feeding substrates with high SO_4^{2-} and NO_3^- contents minimized, for similar reasons. H_2S typically originates from sulfur containing organic matter. Since it is highly toxic for most living beings, maximum working place concentrations have to be respected. Moreover, corrosive acids such as H_2SO_4, H_2SO_3 or HNO_3 can be formed in the presence of O_2 and S- or N-containing compounds. They can damage mechanical devices and constituents of the biogas plant.

2.5 Biological Methane Potential, and Activity, Toxicity and Supplementation Tests

BMP (biological methane potential) or SMA (specific methanogenic activity) tests are typically applied to determine the methane potential of given substrates. By variation of these batch-mode assays in ATS (activity, toxicity and supplementation) tests [39], they can be employed to diagnose and control the actual state of the biogas process. It is attempted to assess e.g. the capacity of inocula to be activated, to evaluate the potential of added or endogenous compounds to exert toxic effects, or to test supplements for process stimulation.

Depending on the type of inoculum and its degree of adaptation, the potential of the biocenosis is tested to produce methane and/or react to changed process conditions. It is emphasized that results of these assays cannot always be used to predict the performance such as the methane yield in flow-through operation. This is mainly due to operational differences, particularly in the effective organic loading rate and the actual microbial retention time. Moreover, these tests are labor- and time consuming, typical test periods vary between several days to weeks.

2.6 Enzyme Tests and Applications

Enzymatic tests are important in research and have some potential for practice application. For example, hydrolytic enzymes are of high interest, since enzymatic saccharification (hydrolysis) is a rate-limiting step in anaerobic digestion (AD) from solid substrates and especially undigested lignocellulosic biomass (LCB) material, e.g. floating layers can pose considerable operational problems [40–42]. Hydrolases must accommodate heterogeneous plant cell wall residing polymers with various degrees of polymerization (DPs), side chain branching patterns and several altering substitutes [43]. Because of this chemical inhomogeneity and substrate specificity corresponding enzymes that act upon them are generally difficult to isolate and characterize. The quantitative determination of enzymatic activities is commonly based on accumulated products after hydrolysis including reducing sugars, total sugars and chromophores. Other assays measure the reduction in substrate quantity or the change in the physical properties of substrates. However, the production of reducing sugars is assayed using alkaline dinitrosalicylic acid (DNS), copper-arsenomolybdate using the 4-hydroxy-benzoylhydrazine (PAHBAH) method, 2,2′-bicinhroninate (BCA) and ferricyanide or directly using anthrone- or phenol-H_2SO_4. Focusing monomeric products, i.e. glucose as major product, commercial enzymatic glucose kits using coupled hexokinase and glucose-6-phosphat dehydrogenase are available. The main drawback of these methods is a poor stoichiometric relationship between reaction products (e.g. cellodextrins, malto- or xylodextrin) and pure D-glucose standards [44], which may result in an underestimation or overestimation of cellulase and hemicellulase activities [45, 46]. However, substrates used for hydrolysis assays should therefore always be as similar to native polymer structures as possible in matters of DP, solubility and crystallinity.

The esterase activity was suggested as indicator for the overall fermentation process, representing a sum parameter for bacterial heterotrophic activity in general [47]. In this context a positive correlation between esterase activity and substrate conversion rate towards methane was observed, revealing that process disruption is reflected by decreased enzyme activities [48, 49]. Furthermore, a negative correlation of esterase as well as aminopeptidase activities and substrate quality was observed, providing fermentability indications regarding silage as substrates [48]. Therefore, enzyme assays can be a useful tool for monitoring the overall anaerobic

digestion process. Modifications towards an all-in-one testing kit like available for other chemical parameters (e.g. Merck Spectroquant® for COD, TOC, nitrate, ammonium etc.) is desirable to provide plant operators with an activity specific easy-to-use monitoring instrument. Compared to photometric tests, lower detection limits (factor 20–500) and shorter reaction times can be reached by the use of fluorimetric determination on the basis of fluorigenic compounds (e.g. fluoresceine diacetate, azocasein) [50]. Thereby, a precise study of catabolic enzyme activities such as esterase, phosphatase, aminopeptidase and glucosidase activities in samples with low biomass density is possible. In sum, non-methanogenic (hydrolytic, acidogenic) and methanogenic activity tests in combination with molecular tools seem to be essential for a better characterization and monitoring of full-scale anaerobic digesters [51].

Beyond measuring the activity or amount of enzymes available in the process, enzymes can be also applied to stimulate the AD process. The utilization of enzymes for environmental and industrial applications have been described to be stable in a large range of even quickly changing conditions, i.e. pH, temperature, presence of inhibitors or interspecies competition [52, 53], although controversially observations have been described by other studies on the use of commercial enzymes and enzyme mixtures considering related costs in AD processes, rather suggesting a specific application with respect to optimum conditions and the source of substrate [54, 55]. It has been shown that a combination of chemical and enzymatic pretreatment of bamboo waste, using commercial cellulase and alkaline, can lead to significantly enhanced chemical oxygen demand (COD) solubilization and substrate saccharification in BMP tests, which not necessarily translates to high methane yields as compared to alkaline pretreatment alone [56], suggesting to re-think the role of enzymes in multiple-pretreatment settings. The application of natural endogenous hydrolases such as amylase and protease from fermentation sludge for pretreatment of wastewater sludge resulted in improved sludge solubilization and acidification regarding the COD and VFA upturn [57], whereas a positive effect on anaerobic biodegradability, hydrolysis, digestion rates as well as maintaining a healthy microbial population were not indicated [58]. However, enzyme treatment can improve the economic production of biogas from agricultural residues, municipal solid and animal wastes by enhancing the fluidity of fibrous feedstock mixtures [59], solubilization and deflocculation of wastewater and sewage sludge biomass towards anaerobic digestibility [60–62].

The instability and time-limited effect of free enzymes can be overcome by immobilization using suitable carriers such as alginate or minerals [52, 63–66]. Moreover, the improvement of high-solid substrate degradation can be also achieved by inoculation of beneficial bacteria, which produce corresponding hydrolases in response to the given feedstock and operating conditions [49, 67, 68]. This has been demonstrated for mixed hemicellulolytic bacteria cultures [69, 70] and isolated bacterial species obtained from natural biogas-producing consortia as well, i.e. hydrogen-producing cultures of *Caldicellulosiruptor saccharolyticus* and *Enterobacter cloacae* [71, 72] or *Clostridium cellulolyticum*, which was

successfully adopted to enhance the hydrolysis of wheat straw leading to increased BMP tests improving the utilization of lignocellulosic substrates [73].

An improved understanding of the catalytic potential of the AD ecosystem can be attained by mechanistic models based on kinetic data capturing important details of enzyme-substrate interactions, key substrate surface properties and individual enzyme adsorption and complexation characteristics as demonstrated for cellulose/cellulase interactions [57, 74, 75]. These catalytic information might be implemented into existing complex dynamic models and simulations such as IWA's Anaerobic Digestion Model (ADM No. 1, 2) or novel Process Simulation Models (PSMs), which are validated against a variety of lab and industrial data on anaerobic digestion to predict the applicability of any substrate for biogas production at any given process condition [76–79].

2.7 Classical Microbiology Approaches

Classical cultivation-based microbiological methods have not gained major importance in the analysis of the biogas process status in practice. This is owed to the fact that most anaerobic microbes have long duplication times. Cultivation of anaerobes is not only tedious and difficult in many instances. It can cause biased results if specific growth and activity requirements of investigated microbes or associations are not known. By applying next generation sequencing (see Sect. 3.1.4), metagenomics, genome analysis and mapping, specific genetic capacities of investigated microbes or associations can be identified which can help to meet unrecognized cultivation requirements and eventually grow hitherto uncultured organisms [80]. Classical light microscopy reveals its limits given the highly turbid sample matrix and the low portion of known and described microorganisms [81].

Bioaugmentation is a classical microbiology measure to counteract process imbalances and a key component of biotechnology routines. Virtually every biogas plant and biotechnological process has been or is started up by inoculation, a special form of bioaugmentation. Numerous experiences show that once a stable process and biocenosis is established, newly introduced strains will encounter enormous difficulties to colonize and propagate in this process [82], although such success was announced in a few reports [71]. Bioaugmentation might be helpful for the case that a disturbed process should be stabilized or re-established by the (re)introduction of certain strains or consortia which had been recognized to be relevant for proper function but were washed out.

3 Molecular Biology Approaches

Biogas process failure can have several reasons. Technical reasons include stirring problems, leakages and temperature changes. At too short microbial retention time and unbalanced or insufficient nutrition (see Sect. 2.4) slowly growing, but possibly important microbes are diluted out. If a process-relevant guild is washed out and no functional substitutes can grow up, this results in process failure or even breakdown. Such bioindicators of the process state are ideally tracked by specific molecular biomarkers. Since these react earlier than the conventional physico-chemical parameters [83], molecular biology bioindicator tracking does not only allow for diagnosing the process, it provides more time to plant operators for specific counteraction.

This chapter is subdivided into several sections where molecular biology methods with more or less potential for process diagnosis are described. Some are used only in basic research and others have started to be applied in practice.

3.1 PCR Based Approaches and Nucleic Acid Sequencing

Since the invention of the Polymerase Chain Reaction (PCR) in 1983, PCR based techniques have conquered the field in molecular biology diagnostics. With the recent progress in affordable next generation sequencing techniques [84] (see Sect. 3.1.4), sequence information in databanks has substantially boosted. On this basis, group-specific primers and probes can be designed with much higher dependability. Diagnostic PCR assays are quickly performed and highly sensitive if suitable (e.g. fluorescence based) detection systems are included. In this chapter, emphasis is therefore on PCR-based methods and among these particularly on quantitative Real-Time PCR (qPCR) assays. PCR applications typically need a specialized laboratory environment but developments for on-site use are emerging. On-line systems, however, are far from being conceivable.

3.1.1 Crucial Prerequisites: Sampling and Nucleic Acid Extraction

A prerequisite for reliable results is that the samples taken are representative of the fermenter sludge. This is not trivial since the fermenter sludge typically is not visually examinable. Bleeders may be partially clogged and act as filters or other phenomena such as floating or sediment layers may cause inhomogeneities. Results should therefore be checked for plausibility and possible sampling bias. Transport and storage of samples is another major source of errors. If samples can be processed within a few hours, they should be kept at process temperature in (almost filled up) closed Polyethylene (PE) or polypropylene (PP) bottles with a cannula for degassing. For longer transport/storage it depends, if DNA as the most stable,

rRNA as intermediate or mRNA as the least stable nucleic acid (NA) is the target [85, 86]. In our experience, samples can be stored at ca. 4 °C for 1–2 weeks for analyses on DNA and for a few days for analyses on rRNA level. It is not finally shown for these NA species whether freezing at −20 or −80 °C respectively, and gentle thawing (at ca. 4 °C) does affect the microbial community composition. For mRNA analysis from stored samples it must be considered that this RNA species is in a highly dynamic equilibrium. Both production and degradation must be stopped immediately, e.g. by immersion in liquid N_2, acid phenol or other effective preservatives. Respective research is currently being carried out.

It must also be shown that the used NA extraction and purification system is suitable and efficient for the specific type of sample and analysis, and for quantitative analyses (see Sect. 3.1.2), the corresponding NA recovery rate has to be known. From numerous comparative studies dealing with NA extraction and purification systems it is turning out that combined physical cell disruption and chemical lysis is most suitable for environmental samples with a high portion of particulate organic matter such as fermenter sludge samples. Washing the sample prior to extraction is suggested because this substantially reduces inhibitors such as water soluble humic compounds [87]. NA purity in extracts is therefore of major concern, but as pointed out below, current guide values are not always conclusive and helpful for PCR-based assays.

Physical disruption of cells to release NAs is another crucial factor for obtaining suitable extracts. Due to velocity, ease of handling and performance efficiency, bead beating (BB) is used most frequently. Rigid cell walls must be broken, but too harsh BB can shear NAs and lead to detection failure [88]. Physical disruption must therefore be optimized for the targeted type of cells along with the particulate organic matter (OM) content in the sample sheltering the targeted cells. The higher the OM content, the more intense BB must be chosen. If differently recalcitrant cells are present, a fractionated protocol with increasing BB force and pooling of sub-sampled extracts can be applied [89]. It is essential to further adapt the protocol to the downstream type of analysis. If relatively short fragments such as for qPCR are suitable, relatively strong BB is of advantage. For applications requiring longer NA stretches such as functional transcriptome or genome analysis, strong BB can be counterproductive.

For RNA extracts, efficient DNase treatment and Reverse Transcription (RT) reaction with -RT controls must be performed, otherwise downstream reactions are contaminated and results biased. It must be considered that DNases degrade RNA to a certain extent, as well. This can introduce uncertainty and may only partially be overcome by method standardization leaving the possibility of a systematic error. The produced cDNA is further used just like genomic DNA but its single-stranded nature must be considered for quantitative aspects.

Downstream, extract purification is a trade-off between inhibitor removal and NA loss. PCR inhibitor removal is frequently seen as equal with matching traditional absorbance ratios (A260/230, A260/280). However, these had originally been developed for DNA-DNA hybridization and turned out to be of limited value for PCR applications. Quantitative Real-Time PCR (qPCR) was not inhibited at

A260/230 and A260/280 ratios as low as 0.02 and 1.4, respectively [32]. Cell-lysing Guanidinium-Isothiocyanat (GITC) present in some NA extraction kits absorbs at 230 nm but does not seem to compromise PCR. However, (partial) inhibition was obtained if the A320 value (humic compounds absorb at 320 nm) surpassed a level of 0.02–0.03. The A320 value thus appears to be a major indicator of PCR inhibition by samples containing humic compounds.

Between 40 % and over 80 % of extracted DNA was lost by conventional silica column post-purification [90, 91], which considerably compromises the sensitivity of quantitative assays. Optimization of the extraction/purification protocol requires that the number of treatment steps is minimized while inhibitor removal and NA recovery rates are maximized. With optimized kit-based DNA and RNA extraction/purification systems and optimized (RT)qPCR biochemistry such as inclusion of a highly processive polymerase and adjusted Mg^{2+} concentration [87], about 90 % of spiked DNA and 30–70 % of spiked viral RNA was recovered from cattle manure or biogas fermenter samples with an optimized kit-based total RNA extraction procedure [83]. However, the RNA recovery rate may actually have been lower because no DNase digestion was performed and DNase I can degrade RNA unspecifically. It is thus strongly suggested to report the method detection limit [91] of the given assay along with the DNA and/or RNA recovery rates.

3.1.2 Conventional and (Reverse-Transcription) Quantitative Real-Time PCR: Applications for Process Diagnosis

Conventional PCR is an integral step of several applications such as amplicon sequencing (see 3.1.4) and community fingerprinting (see Sect. 3.1.3). For diagnostic purposes, however, conventional PCR has lost importance in the last years in favor of Real-Time PCR (qPCR) assays. Applications, advantages and limitations of qPCR and RT-qPCR are compiled and discussed in many reviews and book chapters, e.g. [92–94]. (RT)qPCR assays avoid laborious gel-electrophoresis, are performed more quickly, are suitable for high throughput, are less prone to contamination, and provide superior specificity particularly if an additional (e.g. hydrolysis) probe or different chemistry for the same purpose is integrated [95].

The reliability of PCR assays has significantly been improved. DNA-polymerases with a very low error rate (for *Taq* ca. 3×10^{-5}, still much lower e.g. for *Pfu*, [96, 97]) and suitable reaction environment are available, and primer specificity can significantly be improved due to the enormously grown sequence data in databanks. A major issue, however, is the formation of chimaeras during PCR amplification which can seriously bias community composition analysis. Several programs and online applications can be used to check for chimaeras even in sets with relatively short amplicons [98]. Avoiding the formation of chimaeras, e.g. by analyzing templates with relatively homogenous melting temperatures (T_m) over the region of interest, would be even more straightforward than post-purifying datasets. However, it can be difficult to find template regions that provide sufficient phylogenetic resolution.

Moreover, problems or uncertainty still exist particularly with quantitative analyses of prokaryotic mRNA. This is not only due to unspecific DNase activity (see Sect. 3.1.2), but (partial) inhibition of RT-reactions that typically remain undetected. The RT efficiency at the given reaction conditions typically is not documented and may be subjected to interfering compounds introducing variability. Although these imponderabilia may not be of crucial importance for qualitative approaches such as community analyses, further methodological development is required for reliable quantification of prokaryotic mRNA.

Microbial Guilds, Bioindicators and Transcriptional Profiling

The bioindicator approach (Fig. 1) aims at analyzing and predicting distinct process states, shifts and perturbances, e.g. in biogas reactors. On the molecular level, genes encoding key enzymes of important metabolic pathways in the biogas process such as methyl-coenzyme M reductase (isogenes *mcr* and *mrt* encoding coenzyme-B sulfoethylthiotransferase, EC 2.8.4.1, the key enzyme of methanogenesis, which is present in all and exclusively in methanogenic *Archaea*), formyl-tetrahydrofolate synthetase (or formate-tetrahydrofolate ligase, *fhs*, EC 6.3.4.3, key enzyme of the Wood-Ljungdahl-pathway) or certain hydrogenases (e.g. *ech*, *hyd*) and their transcripts are ideal bioindicators and targets of molecular biomarkers [4], and even more will be identified in the near future [11, 99]. (RT)qPCR markers can be
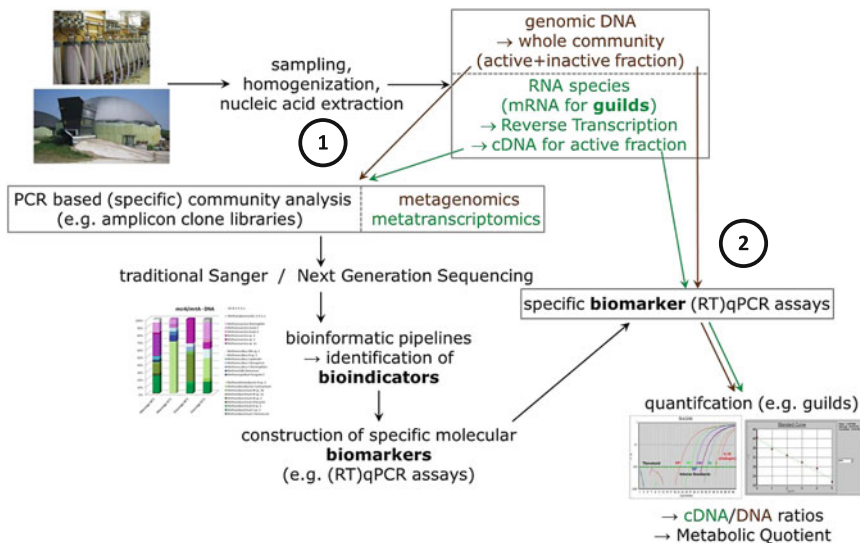

Fig. 1 The bioindicator/biomarker approach to assess process-relevant microbial guilds and their characteristic (transcriptional) activity. Once bioindicators are identified and biomarkers constructed (*1*), guilds and their (transcriptional) activity can be quantified in high-throughput assays (*2*)

tailored to determine bioindicator organisms of such guilds and their transcription activity (Fig. 1). For the design of specific biomarker systems for defined bioindicator organisms, it is essential that these had been identified previously by community composition analyses at relevant fermentation process conditions. However, the design of specific (RT)qPCR systems for heterogeneous microbial groups or clades in environmental samples can be difficult. This is particularly true if guilds are to be tracked by targeting signatures on functional genes exhibiting wobble bases in the third codon position. Respective primers, so-called "protein primers", typically are highly degenerated to provide the desired specificity, which complicates functional PCR based assays (e.g. [100]).

Due to the high stability of DNA, as compared to mRNA (see Sect. 3.1.1), DNA based assays will detect live and inactive organisms as well as residual DNA e.g. of dead organisms [101, 102]. Since for realtime process analysis, the active organisms are of particular interest, transcriptional profiling is supposed to identify more meaningful bioindicators than conventional analyses on DNA level, but changes in environmental conditions can not only induce quantitative transcription changes (see Sect. "cDNA/DNA Ratios and the Metabolic Quotient"). Typically, first metabolic activity and subsequently propagation of the populations that are best adapted to the new conditions are encouraged, whereas unadapted populations are losing competitiveness. The activity of inadequately adapted microbes is cut down first, and subsequently they are diluted out in flow-through processes. In microbial successions initiated by organic loading rate (OLR) increase in biogas processes with renewable resources, different bioindicators of the process status have been identified:

Methanosaeta spp. were present only at long microbial retention times, low acetate and ammonia levels [17, 32, 103] or/and at a feeding regime with a substrate mixture containing manure e.g. from husbandry [104, 105]. A potential bioindicator, tentatively named *Methanosaeta concilii* 2, was identified first from mesophilic maize silage digesters [4]. It is different from the *mcr*A sister clade (*M. concilii* 1) encompassing the type strain and most of the *Methanosaeta* sequences recovered from animal manure environments. Recent sequencing confirmed the presence of *M. concilii* 2 also in mesophilic grass silage digesters. This guild, probably originating from the cattle manure inoculum, soon lost transcription activity in the grass silage digestion process and was washed out subsequently (B. Munk, unpublished) at increased loading rates. *M. concilii* 2 and its activity is thus an example of a specific bioindicator of relaxed digestion conditions.

Other methanogens appear to have a similar potential to be used as indicators of the biogas process status. Results of several studies performed in different environments on the DNA and on the transcription level [4, 83, 106–108] suggest that with increasing strain to stress conditions such as shorter microbial retention times with increased SCFA concentrations and critical ammonia contents, *Methanosaetaceae* and their activity are replaced by *Methanosarcinaceae* and *Methanobacteriaceae*, with the latter appearing to be the most resistant. More specifically, certain *Methanosarcina* genospecies, hitherto undescribed *Methanosarcinaceae* (tentatively classified as genus II) and strictly hydrogenotrophic methanogens, particularly *Methanobacteriaceae*, certain *Methanobacterium*

genospecies and hitherto undescribed *Methanobacteriaceae* (tentatively classified as genus IV) were increasing in maize and grass silage digestion processes on the DNA and on the transcription level [4, 83]: at aggravated strain or stress conditions, very short retention times, high SCFA or ammonia contents, at the onset of process failure, the diversity of methanogens remained almost unchanged at the DNA level, but *mcr*A/*mrt*A was transcribed exclusively by certain *Methanobacterium* genospecies (particularly *Methanobacterium* III sp. 3a) at mesophilic and *Methanothermobacter wolfeii* at thermophilic conditions. For these bioindicators, more specific (RT)qPCR based biomarker systems are being developed in order to track their presence and activity and provide a meaningful process diagnosis.

cDNA/DNA Ratios and the Metabolic Quotient

In principle, cDNA/DNA ratios can be calculated for any physiological performance of interest by relating the actual net concentration resulting from RNA transcription and transcript degradation to the concentration of the corresponding gene in a given sample. It appears to be most meaningful to determine the cDNA/DNA ratio of selected functional genes of key enzymes as activity parameter to assess the specific activity of certain guilds (see Sect. "Microbial Guilds, Bioindicators and Transcriptional Profiling", Fig. 1). Respective necessary information for designing specific (RT)qPCR system can be derived from alignments containing relevant sequences deposited in databases and extracted sequences from metagenomes and metatranscriptomes (see Sect. 3.1.4).

For *mcr*A/*mrt*A, cDNA/DNA ratios have already been reported, e.g. for peat soil and biogas fermenters [31, 109, 110]. The cDNA/DNA ratios reacted to activating stimuli such as temperature or substrate, whereas the gene concentrations remained almost constant, and they were correlated with the methane production rate within certain limits, indicating the potential of this molecular biology approach to track the activity of the guild of methanogenic *Archaea*.

Similar approaches may be envisaged to track distinct microbial activities. Concerning biogas processes, cDNA/DNA ratios, e.g. for *fhs* or *ech* subunits or other important genes of key enzymes could be very informative on the activity status of the corresponding metabolic pathways. Such information would be very helpful for process diagnosis also for e.g. biorefineries, and monitoring could provide operators with necessary information for process engineering and to decide on possible intervening measures.

However, it has to be considered that prokaryotic mRNA analysis still is delicate and error-prone, particularly if quantitative results are to be obtained (see Sects. 3.1.1 and 3.1.2). At the current state of the art, respective results should therefore be treated with precaution. RTqPCR and upstream sample preparation still need methodological development until interlaboratory comparison will create consistent and reliable results.

A second ecophysiological parameter is the Metabolic Quotient (MQ). The MQ has been developed by Munk et al. [31] and was further explained in more detail

[4]. In contrast to the entirely molecular biological parameter cDNA/DNA ratio, the MQ needs concomitant physiological data. For the MQ, the methane productivity (mL CH_4 per mL fermenter sludge) is related to the concentration of methanogenic *Archaea*, as determined by *mcr*A/*mrt*A targeted qPCR [100] on the DNA level, regardless if they are dead or alive, in the fermenter sludge at a given time, resulting in the actual specific methanogenic activity (SMA_{act}). SMA_{act} is compared to a reference standard dataset (SMA_{std}) obtained for efficient process performance at various OLRs without any symptoms of process disturbance. If SMA_{act}/SMA_{std} is >1, the methanogenic guild of interest is metabolizing at strain or stress conditions, and if SMA_{act}/SMA_{std} is <1, the methanogens are less active than at the standard reference conditions.

The MQ was measured in different maize silage digestion processes along with conventional indicators of the process state (see Sect. 2) in time series [31, 83]. It turned out that the MQ passed a threshold of about 3 ca. 2 weeks before changes were detected by the conventional chemical process indicators such as noticeable increases of the TVA/TIC ratio or SCFA concentrations. At this process stage, less methanogens than at standard conditions performed the same metabolic task, indicating metabolic strain or even stress of the given methanogenic population. When the TVA/TIC ratio and/or critical SCFAs such as propionic acid had increased to an alarming level of about 0.7 or 1 g/L, respectively, the MQ began to decline or had already decreased, indicating serious process failure and collapse. The methanogenic population was obviously seriously affected and not able anymore to fully accomplish the metabolic task of methane formation, as evidenced by the sudden decrease of methane productivity and the methane yield. When no substrate was fed to the process, the MQ was significantly below 1. According to the observations, a threshold of ca. 0.1 was defined, indicating the lowest level of normal physiologic activity.

The MQ thus allows, over the complete range of tested OLRs, to determine the metabolic state of the resident methanogenic population. A single MQ determination, however, does not necessarily mean very much. Just like with the TVA/TIC ratio, the recent development has explanatory power. An increasing MQ indicates increasing strain or stress. A decreasing MQ can indicate relaxed conditions or process breakdown. If an MQ of 1 was measured, it can be a sign of normal process operation, but it can also be symptom of a collapsed process if it turned down from values exceeding 3.

In most recent experiments with grass silage as substrate and measurements of practice biogas plants operated predominantly with grass silage, the MQ reacted similarly as in maize silage processes and stood within the bandwidth of 0.1–3 at normal process conditions without symptoms of process disturbance (B. Munk, personal communication). Since the TVA/TIC ratio is losing informative value at the high ammonia contents typically found in grass silage digestion, this is of particular importance and demonstrates the potential of the MQ as an early warning tool of process failure in practice (Fig. 1). It is expected that the MQ will become an important ecophysiological molecular microbial parameter and find application in practice monitoring of biogas plants and process diagnosis.

3.1.3 Community Fingerprinting Assays

An ideal method for microbial community analysis would allow the detection of different groups and enumerate all microbial species present in a sample from an ecosystem or habitat. Basically two approaches are used for community analyses: (1) cultivation-dependent analysis (CDA) aiming at the detection of selected groups and species of microorganisms and (2) cultivation-independent analysis (CIA), which are RNA/DNA based and are used to assess the complexity and dynamics of microbial communities. CDA relies on several selective and non-selective culture media that supply different growth conditions for specific or non-specific microbial population targeting. Traditional methods require a vast knowledge of phenotypic features to characterize microorganisms, which is often inaccurate and also leads to an underestimation of the diversity of species. However, the main drawback of conventional cultivation methods to recover less than 1 % of the total microbial species present in environmental samples remains problematic [111]. Thus CDA is nowadays complemented by molecular methods such as polymerase chain reaction (PCR) and fingerprinting techniques to assess shifts in microbial composition by small subunit ribosomal RNA gene analyses [112–114]. CIA is principally based on molecular techniques (Table 1), applying PCR and oligonucleotide probe

Table 1 Molecular screening techniques for microbial community detection, fingerprinting and identification according to [112, 119], modified

Method	Principle	Application	References
AFLP	Restriction of total microbial DNA	Strain-level identification	[120, 121]
ARDRA	Restriction of rRNA genes	Strain-level identification	[122]
ARISA	Automated riobosomal intergenic spacer region length differences (multiple ISRs per genome)	Species-level identification	[123]
RAPD	Random amplified polymorphic DNA	Strain-level identification	[124, 125]
rep-PCR	Repetitive element sequence-based PCR (short sequence repeats [SSR])	Strain-level identification	[126]
LH-PCR	Length heterogeneity of PCR-amplified rRNA genes	Community analysis	[127]
PFGE	Genomic restriction fragments in pulsed-field gel electrophoresis	Strain-level identification	[128]
T-RFLP	Terminal restriction fragment length polymorphism of rRNA genes	Strain-level identification	[129, 130]
DGGE	Mobility of partially melted dsDNA in linear gradient of DNA denaturants	Community analysis	[131,132]
TGGE	Mobility of partially melted dsDNA in linear temperature gradient	Community analysis	[133, 132]
SSCP	Mobility of conformed ssDNA in non-denaturing gels	Community analysis	[134, 135]

hybridization in order to identify microbes directly from sample material [115]. Therefore, total genomic DNA or RNA must be extracted from collected microbial cells, avoiding co-extraction of sample matrix-inherent compounds that can totally inhibit the PCR (see Sect. 3.1.1). Cell lysis is accomplished by several methods: mechanically using bead-beating, freeze-boil cycles, chemically by the use of detergents or enzymatically using cell wall degrading enzymes, e.g. lysozyme, lyticase or proteinase [116–118].

Environmental microbiological studies are often based on ribosomal DNA or RNA sequences, because these sequences are functionally and evolutionary conserved and present in all organisms. Here, 16S rDNA and 23S rDNA sequence regions have already been determined for a large number of reasonably described bacterial, archaeal and fungal species. Thus 16S rDNA sequences can be used to investigate phylogenetic relationships and for the identification of unknown microbes via comparisons with database collection entries. The largest reference databases exist for conserved marker gene 16S rRNA [112, 136]. In contrast to rDNA, rRNA targeted techniques rely on high-copy numbers per cell and are specifically used to assess changes in metabolically active microbial populations [137], although extraction and handling procedures are much more complicated due to the rRNAs instability (v. [138]). The intergenic spacer region (ISR) between 16S and 23S rDNA often shows species specific sequence variations by primers binding to conserved nucleotide stretches at the 5′ 23S and 3′ 16S rDNA gene end respectively [139]. In ISR-directed ribosomal intergenic spacer analyses (RISA) it is used to describe phylogenetic microbial diversity (*Bacteria* and *Archaea*) by creating RISA profiles. Although it is foremost used in diagnostic PCR-amplifications [140], ISR amplicons as targets for qPCR assays have also been discussed to reflect the metabolic status of key microbes more accurate than 16S based fragment comparisons [141]. Numerous broad-range and group-specific primers are available, targeting many bacterial and archaeal species of interest in AD processes, including fermentative and methanogenic representatives, covering low diversity selective cultivation sample structures up to full-scale agricultural biogas plant complex mixed community fingerprints [24, 69, 142].

The fingerprinting techniques range from simple length heterogeneity PCRs (LH-PCR) depending on different primers, targeting several variable regions in combination [143] up to more sophisticated genetic fingerprinting techniques such as amplified ribosomal DNA restriction analysis (ARDRA) or terminal restriction fragment length polymorphism (T-RFLP), which are all well established and vastly exploited to characterize whole microbial communities, providing pattern profiles of the community diversity [129, 144, 145].

T-RFLP fingerprints can give quantitative insights into communities by using a combination of fluorescence labelled primers and enzymatic digestion of resulting PCR products to generate terminal restriction fragments (T-RFs) from DNA templates. The taxonomic resolution can be improved by combining several fluorochromes and restriction enzymes simultaneously [146, 147]. Problems ascend from incomplete restriction digestion due to e.g. missing restriction sites or fragment length discrepancies caused by different fluorochromes used to estimate in silico

yields, which reduces the reproducibility [130]. However, T-RFLP has not only been applied to describe bacterial communities, but also to monitor methanogenic populations and temporal shifts of archaeal communities in bioreactors [148, 149].

Further fingerprinting techniques are denaturing-gradient gel electrophoresis (DGGE), temperature-gradient gel electrophoresis (TGGE) and single-strand conformation polymorphism analysis (SSCP), which detect sequence variations of rRNA gene fragments or other functional genes from total community DNA or cDNA [150]. Complex microbial communities can thus be resolved into single members through band separation by gel electrophoresis. DNA sequence information is obtained from excised bands, which represent operational taxonomic units (OTUs) or even single species, but do often require further preparation and time consuming cloning steps. Co-mitigation or poor separation of bands representing small fragments less than 500 bp and restricted sensitivity to OTUs with a minimum abundance of 10 % (SSCP) lead to limitations of phylogenetic identifications and incomplete microbial profiles [151], which can be partly overcome by e.g. nested PCR, widening the spectrum of detectable phylogenetic groups in direct comparison to dominant members of the bacterial community [152]. In order to reach higher throughput numbers than Sanger Sequencing can provide at this stage, next generation sequencing technologies are used alternatively to analyze thousands of OTUs from different functional guilds. Yet, for complex environmental samples such as soil samples, DGGE, SSCP as well as T-RFLP provide similar compelling results on bacterial community composition [153] and microbial dynamics [152, 154], but a major drawback of ribosomal DNA based fingerprinting methods is that all DNA present in a sample is amplified, regardless the metabolic activity of bacteria, thus being less usable to reflect acute process dynamics such as crises in anaerobic digesters alone (see 3.1.2). Furthermore, molecular fingerprinting methods are not considered quantitative, but can include quantitative matrices as basis for dendrograms or can be related to multivariate analyses including process parameters, hierarchical clustering and specific microbial activities should be combined with genomic/fingerprinting data and incorporated into multivariate ordination methods such as Principal Coordinate or Principal Component Analysis (PCoA/PCA) in order to complete the whole picture drawn from a biogas biocenosis [26, 112, 155].

To evaluate environmental and process derived ecosystems, diversity is a suitable parameter that is measured by the number of different species (also from phylogenetic identification of OTUs from clone libraries assuming that one OTU corresponds to one species) including the inequality in relative abundance (Fig. 2). Therefore, diversity indices include abundance, richness and evenness as well as the Shannon index (H'). Abundance is the relative representation of a species in a community, i.e. number of a specific organism. Richness is defined as the number of different species or OTUs obtained from fingerprinting or cloning methods. Evenness is a measure of the equitability of abundance. The Shannon index (H') is calculated by the relative abundance and richness of each species (OTU or T-RFLP peak) respectively. The higher the number of phylotypes evenly distributed, the higher the H' index, which is specifically appropriate for the evaluation of low
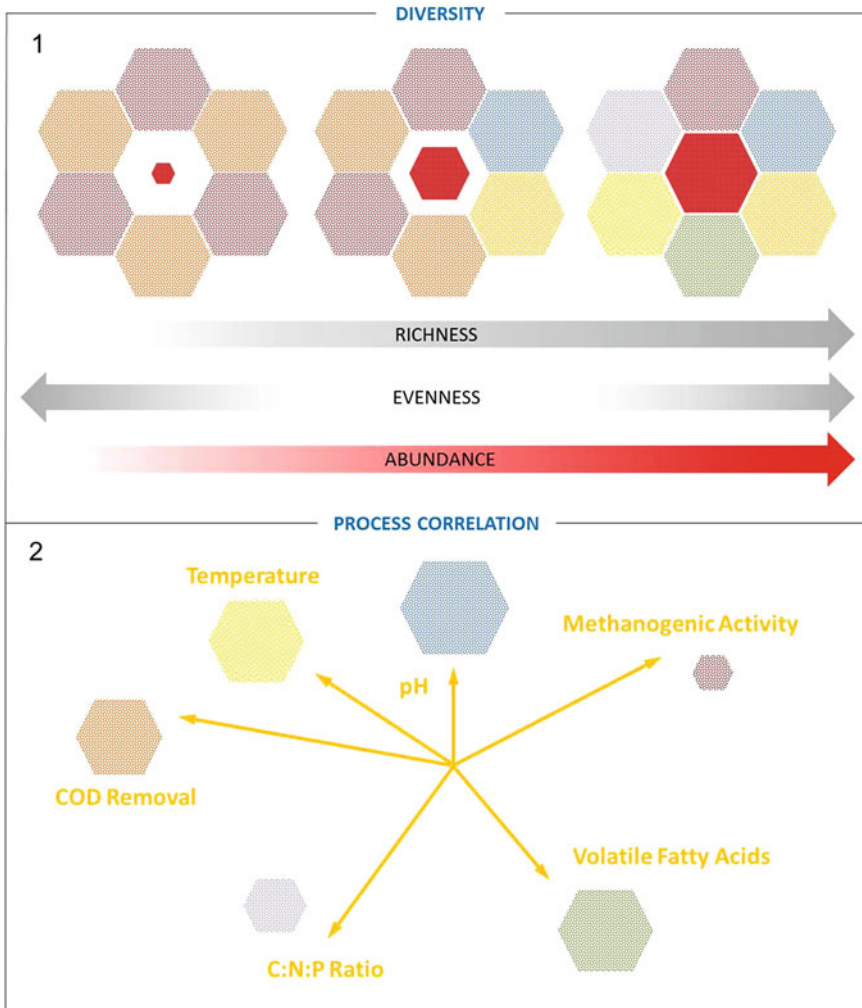
Fig. 2 Diversity—**a** complex parameter to describe the microbial composition of a given ecosystem defined by the indices richness (increases with the number of different species), evenness (distribution of present species) and abundance (number of a certain species), each colored hexagon represents one species (*1*); linking community data with process parameters, different hexagon sizes reflect the number of each species (abundances) ideal-theoretically correlated with typical process parameters and their occurrences (*2*)

abundant, but important species (indicator species) due to its high sensitivity by proportional weighting [156]. Recent community studies regarding mesophilic and thermophilic co-digestion [157] and CSTR feeding pattern comparisons [158] indicate that high initial evenness (more dynamic populations) favors the microbial functionality under selective stress conditions, suggesting the microbial community to be more flexible.

To include activity analysis, microarrays based on the hybridization of oligo-nucleotides or PCR products can be used to generate gene expression profiles and signatures and have been applied to investigate bacterial communities of composts as well as methanogenic communities by specifically designed microarray-chips, i.e. COMPOCHIP and ANAEROCHIP [159, 160]. This technique has also been applied in combination with real-time PCR to investigate and quantify specific targets of organic waste associated microbial communities [161]. However, the traditional microarray approach cannot detect novel genes since the device construction only involves known nucleotide sequences [162]. Therefore, metatranscriptomics described in the following section are currently preferred to enable gene expression identification without a priori sequence knowledge [162, 163] (see 3.1.4).

3.1.4 Next Generation Sequencing and Meta-Omics

The advent of affordable high-throughput Next Generation Sequencing (NGS) [84, 164] has boosted the number of sequence entries in databanks. This information has not only significantly enlarged our knowledge in systems biology; it represents an invaluable basis for further developments and exploitation. Different NGS technologies using emulsion or bridge PCR are available and can generate millions of parallel reads in small volume reactions with average read lengths between ca. 40 and about 1,100 bp. Illumina platforms are currently the most frequently used. 454 pyrosequencing will no longer be sustained. Originary Pacific Biosciences' (PacBio) RS sequencers typically generate long reads of >1 kb but the cost per base and the raw error rate (>10 % on average) are relatively high. Cost and error rates are actually lowest for Illumina and in between for Ion Torrent PGM systems [164, 165], but PacBio RS sequencing can be particularly useful e.g. by resolving problematic genomic areas such as AT-rich regions.

As compared to Sanger sequencing, error rates of these NGS systems are high, and problematic (GC-, AT-rich) regions can cause bias. Since this can result in erroneously high diversity, as observed in some ecosystem analyses, high coverage of parallel reads is required to generate reliable NGS results. Including data processing, particularly sequence assembly, all of these issues necessitate massive biocomputing efforts [166]. Respective bioinformatics pipelines and their maintenance are not affordable for any lab. Many limitations, however, will soon be overcome. Cheap annotation via cloud computing is already feasible [167], and developments towards increased read length and accuracy are going on. For example, PacBio recently introduced the RS II sequencers which are based on single molecule, real-time (SMRT) technology. PacBio claims that half of the data are in reads >14,000 base pairs with accuracy equal to Sanger sequencing. In a recent report (Mosher et al. 2014), PacBio RS II sequencing using P4/C2 chemistry surpassed the accuracy of Roche/454 pyrosequencing and generated longer reads.

For application of PCR based NGS approaches, it must additionally be considered that such amplicon sets are typically interspersed with chimaeras (see Sect. 3.1.2). These dissemble higher diversity than actually present and must be eliminated [98]. In addition, possible bias associated with Reverse Transcription or DNase treatment (see Sect. 3.1.1) must be considered [168]. However, if these challenges are adequately met, amplicon sequencing and metagenomics can produce highly comparable results, as this was shown for samples from different biogas processes by parallel analyses of curated V6-V8 (similarly as the V3-V5) 16S rDNA amplicon libraries and extracted 16S rDNA sequences from metagenomes (without interspersed selective PCR step) [169]. Although the NGS sequence numbers were much higher in this comparison, the PCR approach that was directed against the highly variable V6-V8 region provided substantially more profound insight into the bacterial community structures, occasionally even below the genus level.

NGS analysis of (complete) microbial genomes is another approach of inestimable value. It not only deepens our knowledge on microbial capacities, with the rising number of sequenced genomes and improved annotation, a more and more solid reference database is created for metagenomics and metatranscriptomics [170], leading e.g. to more reliable reference matches and improved binning accuracy. Metatranscriptomics currently is the most straightforward approach to investigate (key) metabolic pathways of interest at the transcription level, and RNAseq-based approaches allow quantitative transcriptome profiling, if suitable reference genomes or transcriptomes are available (Mutz et al. 2013). Although transcriptional activity is mostly regarded as equivalent with expression and activity, subcellular compartmentalization or excretion of enzymes and regulation are occurring at the protein level, and posttranslational modification can alter protein location and function. Additionally cross-linked metabolomics and metaproteomics might thus better reflect functional protein expression and activity in future (Vanwonterghem et al. 2014).

Although all of these approaches are providing an increasingly indispensable information background and data mining repository, they will not be applied for production scale monitoring and real-time process assays since equipment costs are too high for routine analysis, and highly skilled personnel is required. However, based on the compiled background, more meaningful and informative bioindicators may be identified and respective specifically targeted, e.g. (RT)qPCR based biomarkers could be developed (see Sect. "Microbial Guilds, Bioindicators and Transcriptional Profiling"). Such assays are much better suited for labs performing routine analyses.

3.2 Microscopy Based Detection of Microorganisms: Specific and Non-specific Imaging

For observations of bacterial and archaeal cells and biofilms, granules or flocs several microscopy techniques are useful, reaching from simple light microscopy (LM) with limited resolution to high-resolution scanning electron microscopy (SEM), transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM). Amongst them, fluorescence-coupled microscopy is highly sophisticated due to its ability to detect selected groups or specific species within complex mixed communities. It is therefore widely used in microbial ecology studies allowing the visualization of spatial distribution of cells in a sample. For an in situ hybridization, a labelled probe, i.e. a fluorochromes or radioactive signal joined denatured DNA fragment is annealed to a sequence homologous to a certain target DNA (genomic DNA or PCR-amplicons). Using group- or species-specific staining, the differentiation between distinct populations is permitted leading to deep insights into the organization of biofilms and flocs [171], but strongly depends on the type of microscope used.

3.2.1 Fluorescence in Situ Hybridization-Based Confocal Laser Scanning Microscopy (FISH-CLSM)

Whereas epifluorescent imaging gives optical information from only one layer in two-dimensions [172], confocal scanning laser microscopy (CLSM) is capable of imaging a specimen via successive expositions of thin sections that can be reconstructed by computational assistance for 3D and 4D image visualization and analysis (IMARIS, Bitplane, Oxford Instruments). This allows the determination of multi-dimensional relationships of cells and their surroundings [173, 174]. The specimen is focused with a laser beam and pinhole selected fluorescent signals are detected by a photomultiplier, which results in high sensitive, high detailed and non-destructive image acquisition [175]. Fluorescence in situ hybridization targets genera or species specific ribosomal RNA fragments via probes available for *Eubacteria* (EUB) and *Archaea* (ARC). These fragments are specifically labeled with fluorescent dyes (Cy3, Cy5, FITC or FLUOS) that have individual emission wavelength optima to detect and identify multiple populations of target organisms in one sample at the same time [176]. A vast assortment of organism specific probes has already been described [177–179] and the list is constantly expanding in databases such as 'probeBase' [180], which provides currently over 1,300 rRNA-targeted oligonucleotide probe entries. The FISH-CLSM derived image also allows the rapid quantification of fluorescence signals, i.e. number of specific cells or percentage of area covered by biofilms [181]. Minimal statistical evaluation requires three independent samples and the observation of three individual specimen spots, when samples are homogeneous and evenly distributed [182]. At this juncture, flow cytometry (FCM) combines the advantages of microscopy and

biochemical analysis for the measurement of biochemical and physical characteristics of individual cells moving in a fluid stream passing an optical sensor [177, 183, 184] (Fig. 3). In this regard, cytometric fingerprints have been reported to enable the decoding of microbial community dynamics in managed anaerobic microbial systems [185]. CLSM can also be combined with Raman spectroscopy to e.g. examine extracellular polymeric substances (EPS) producing biofilms and thereof distributed polysaccharides such as cellulose, alginate, sodium alginate, dextran, or nucleic acids during the development of the whole biofilm [186].

Numerous fluorescent dyes for DNA or RNA specific staining such as acridine orange or 4′,6-diamidino-2-phenylindole (DAPI) are used in addition to probe specific labeling, e.g. to assess the total number of bacteria against specific signals from fluorescein-labeled species [187]. Commercially available viability kits for fluorescence microscopy, e.g. Live/Dead BacLight[TM] (Molecular Probes®, Life Technologies) can be used to discriminate between viable and non-viable cells. Furthermore, a broad range of fluorescein-coupled molecules such as polyanionic dextrans or lectins of various molecular masses, redox-sensitive chemical probes (e.g. resofurin and fluorescein) and other fluorogenic substances (e.g. fluorescein diacetate) can be used in live cell imaging experiments to analyze (i) chemical interactions of defined molecules, (ii) cellular physiological conditions about
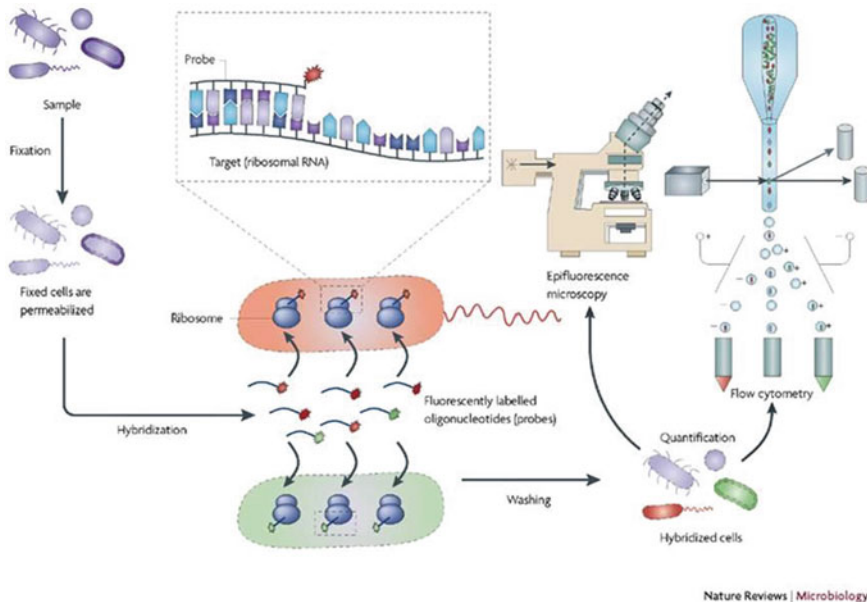
Fig. 3 Single-cell identification and quantification by either epifluorescence microscopy or quantitative flow cytometry on the basis of fluorescence in situ hybridization according to Amann and Fuchs [177]. The sample preparation involves the fixation of microbial cells to stabilize and permeabilize their membranes to allow labelled oligonucleotide probes to access and hybridize to certain intracellular targets. Adapted from Jul 20, 2015, Nature Publishing Group

membrane potential or permeability and (iii) microzonal variations in biofilm chemistry regarding pH, redox potential or ion concentrations [188–190]. FISH is also performed in combination with fingerprinting methods (see Sect. 3.1.3) or cloning experiments as full-cycle rRNA approach to quantitatively determine the relevance and spatial distribution of given operational taxonomic units (OTUs) [191].

Microautoradiography-coupled FISH (MAR-FISH) is another tool for structure and function analyses in microbial ecology [192] that links phylotypic characteristics with metabolic activities to reveal microbial species responsible for key physiological processes [193, 194]. The microbial in situ uptake and incorporation of radioactively labelled substrates can be visualized and enumerated this way [195], but the method is limited to elements with radioactive isotopes (e.g. $^{13/14}$C, ^{3}H, ^{15}N, ^{34}S, ^{33}P, ^{18}O), which makes secondary ion mass spectrometry (SIMS) become a constitutive alternative for MAR-FISH. However, in anaerobic digesters it has been used to elucidate metabolic functions of minor phylogenetic groups like *Chloroflexi*, *Syntrophomonas Spirochaeta* and *Synergistes* as well as *Methanosaeta* spp. in sugar and short fatty acid such as acetate, butyrate, and propionate utilization [196, 197], and led to the determination of degradation rates of glucose, acetate and propionate as well [198, 199]. Although not providing quantitative data, the complementary combination of MAR-FISH with quantitative real-time PCR can be useful to investigate active key functional microbial groups [112].

FISH probing and CLSM have thus been used to show, how microbial communities involved in the anaerobic biodegradation process are organized regarding biofilm formation, immobilization and attachment to solid substrate material [64, 200–202], but also to study bioreactor and full-scale biogas plant performances [203–205]. There are some drawbacks using hybridization based fluorescence microscopy that include fading or photo-bleaching of the fluorochromes, fluorescence quenching, the loss of fluorescence due to sample derived molecules interacting with the fluorochromes, limited archaeal cell wall permeability and inefficient or incorrect hybridization. Many of these problems can be overcome by modifications of the preparation protocol towards sample and organism (i.e. gram-positive/gram-negative *Bacteria* and methanogenic *Archaea*) optimized hybridization conditions such as temperature or formamide concentrations [206], enzymatic pretreatment as routinely applied for catalyzed reporter deposition FISH (CARD-FISH) [207], up to double labeling (DOPE-FISH) for improved signal intensity and rRNA accessibility [208, 209], or even individual probe design [177, 210]. FISH-CLSM is clearly a valuable technique for AD processes to analyze microbial dynamics, since both, qualitative and quantitative information can be obtained, but specialized personnel and laboratory equipment is required to perform these analyses.

3.2.2 High-Resolution Microscopy: Scanning Electron Microscopy (SEM)

Apart from molecular biology depending light microscopy techniques, biofilms and single cells can be also investigated by scanning electron microscopy with unequalled magnifications of up to 500,000-fold (Carl Zeiss Ultra 55, Hitachi S-3000 N). New microscope-generations like RISA even integrate correlative, confocal Raman imaging with scanning electron microscopy (Raman-SEM), permitting a direct link between ultra-structural surface properties and molecular compound information (WITec, TESCAN). Therefore, scanning and transmission electron microscopy (SEM/TEM) have not only been used to study overall biofilm organization patterns (see below), but to investigate cell-to-cell interactions of anaerobic digestion process innate syntrophic microbial partners on a nano-scale level such as the interspecies electron transfer [211]. Direct interspecies electron transfer (DIET) depends on hydrogen and carbon source such as ethanol or formate, which was recently discovered for *Geobacter metallireducens* and *Methanosaeta harundinacea* or *Methanosarcina barkeri* interactions to lastly reduce carbon dioxide to methane [212]. It has also been shown, that growth of fermentative and methanogenic microbes on conductive carriers is tangible, suggesting *Bacteria* (most likely Clostriaceae) and methanogenic *Archaea* (most likely Methanobacteriaceae) can transfer electrons from a stainless steel support even without the involvement of hydrogen or formate [213].

Considering the low growth rate of methanogenic *Archaea*, immobilization on support material such as polymers (e.g. polyurethane, acrylonitrile-acrylamide, nylon) is a potential strategy to allow longer residence times in bioreactors for the adjustment to unstable conditions and varying feeding regimes [214] as shown by SEM for lab-scale reactors continuously operated with vinasse waste to keep COD removal rates constant at decreased retention times and various organic loading rates [215]. SEM was also used to study the natural biofilm formation on zeolite particles during *in sacco* incubation in semi-continuously, completely stirred lab-scale fermenters fed with grass silage [64, 202], or comparing several other carriers for *Bacteria* and *Archaea* such as activated carbon, polyvinyl alcohol or glass fibers in anaerobic digesters treating cattle manure [216], demonstrating that specific materials can selectively support methanogens to avoid co-cultivation of unwanted sulfate-reducing bacteria (SRB) during anaerobic wastewater treatment and methane production from molasses [217]. Focusing feedstock for AD, SEM can be part of efficiency evaluations of pretreatment methods for specific substrates such as that steam explosion induces significant morphological changes in treated lignocellulosic materials [218, 219].

In addition to the direct observation of sputtered organic matter, energy dispersive X-ray spectroscopy (SEM-EDS/EDX) allows element analyses of inorganic sample components of carrier materials or to characterize stable and active catalysts for hydrogen production from biogas, using SEM-TEM in combination with other microscopy methods to evaluate the deposition or arrangement of hollow carbon nanotubes and nanofibers [220]. Furthermore, EDS and TEM can be used for the

localization of substrates or electron donors and acceptors or characterization of e.g. metal transformation in metal-reducing bacteria. However, the major drawback of electron microscopy is that it is an invasive method, which requires sample fixation and preparation including consecutive dehydration steps for specimen observations in high vacuum. Biological structures can be maintained by critical point drying, lyophilisation or high-pressure freezing. Instead of SEM, environmental SEM using lower vacuum pressures can be used alternatively as well. Pinpoint extraction and ultra-thin layer observations by consecutive cryosectioning are further techniques to investigate certain regions of interest and cellular aspects respectively based on SEM/TEM or focused ion beam (FIB)-SEM that can be also combined with CLSM 3D imaging for real 3D correlations of one and the same biological event in an identical sample [221, 222].

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